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Virus-like Particles Templated by DNA Micelles: A General Method for Loading Virus Nanocarriers

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1. List of Abbreviations

AFM	Atomic force microscopy
AIEX	Anion exchange
CP	Coat protein
DiI	1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DLS	Dynamic light scattering
FPLC	Fast protein liquid chromatography
MALDI-TOF	Matrix-assisted laser desorption/ionisation time-of-flight
MWCO	Molecular weight cut-off
PAGE	Polyacrylamide gel electrophoresis
PPO	Poly(propylene oxide)
TEM	Transmission electron microscopy
THF	Tetrahydrofuran
Tris	Tris(hydroxymethyl)aminomethane
VC	Virus capsid

2. Materials and Techniques

Solvents and reagents for DNA synthesis were purchased from Novabiochem (Merck, UK) and SAFC (Sigma-Aldrich, Netherlands). Solid supports, Primer SupportTM (200 μmol / g) from GE Healthcare, were used for synthesis of DNA materials. ÄKTA oligopilot plus (GE Healthcare) was used for DNA synthesis. ÄKTA explorer (GE Healthcare) was used for purification and analysis of DNA materials. After synthesis DNA amphiphiles were purified by anion exchange chromatography, HiTrapTM Q HP 1 ml or 5 ml column (GE Healthcare) through custom gradients using elution buffers (A: 25 mM Tris, B: 25 mM Tris and 1.0 M NaCl). Fractions were further desalted by either desalting column (HiTrapTM desalting, GE Healthcare) or dialysis membrane (MWCO 2000, Spectrum[®] Laboratories).

Molecular weights of **UU11** were determined by MALDI-TOF mass spectrometry. The spectra were recorded on a Bruker MALDI-TOF (Reflex-TOF) mass spectrometer.

The oligonucleotide (5'-AAGACGCCAAA-3') modified with 6-Carboxylic-X-rhodamine (ROX) ($\epsilon = 80,000 \text{ cm}^2 \text{ M}^{-1}$) at the 5'-end was purchased from Biomers, Germany.

AFM images were collected with a MultiMode-II AFM connected with Nanoscope IIIa controller (Veeco) in a fluid-cell filled with milli-Q water, filtered with 0.2 μm syringe filter prior to use. V-1 grade mica plates were purchased from Electron Microscopy Sciences (USA). Silicon nitride cantilevers with silicon tips and spring constant of 0.32 N/m (SNL-10) were purchased from Veeco (France).

FPLC measurements were performed using a Superose 6 PC 3.2/30 analytical column from GE lifesciences, on an Amersham Ettan LC system, fitted with a fractionating device. Buffers for FPLC were filtered with a Millipore 0.2 μm filter before use.

TEM grids (Formvar-Carbon) were exposed to an electron discharge treatment using a Cressington Carbon coater and power unit. The sample was applied to the grids by adding a 5 μL drop of sample solution ($\sim 0.2 \text{ mg/mL}$) to the grid and carefully removing it after 1 minute immersion using a filter paper. The grid was allowed to dry for at least 15 min. before applying 5 μL of a 2% (w/v) uranyl acetate aqueous solution, which was removed after 15 s. The grid was again allowed to dry for at least 15 min. Samples were studied on a JEOL JEM-1010 TEM (Jeol, Japan).

DLS measurements were performed on a Zetasizer Nano S and Zetasizer 5000 (Malvern Instruments Ltd, England) with 90° scattering angle and analyzed using CONTIN.

Protein concentrations were determined with a Cary 50 Conc (Varian, Middelburg) UV-VIS spectrophotometer using a quartz cuvet with a path length of 3 mm.

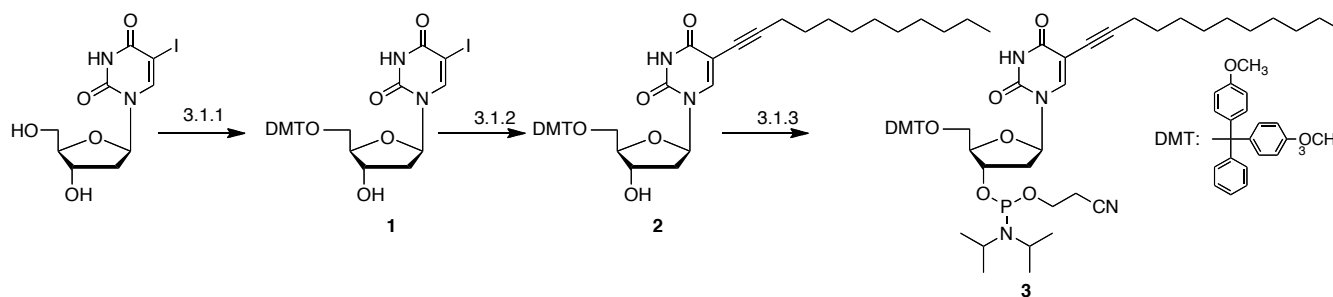
A capsid buffer pH 7.5 (0.5 M NaCl, 0.05 M Tris-HCl, 0.01 M MgCl_2 and 0.001 M EDTA) was used in all VC formation experiments.

2.1. Preparation of CCMV coat protein

The purification of the CCMV virus and the removal of its RNA were carried out according to literature procedures.¹ CP was labeled with Dylight-647 ($\epsilon = 250,000 \text{ cm}^{-1}\text{M}^{-1}$) following the procedure outlined in the product manual, in 17% of coupling efficiency.

3. Synthesis and Characterization of UU11 and P11/P22

3.1. Synthesis of the modified uridine phosphoramidite



Scheme S1. Synthesis of the modified phosphoramidite (3).

3.1.1. 5'-O-(4,4'-dimethoxytrityl)-5-iodo-2'-deoxyuridine (1)

5-Iodo-2'-deoxyuridine (5 g, 14.12 mmol) was dissolved in 80 ml pyridine and reacted with 4,4'-dimethoxytrityl chloride (DMT-Cl) (5.76 g, 17.01 mmol) overnight at room temperature. Afterwards, ice-cold water (50 ml) was added to the solution and the resulting mixture was extracted twice with 50 ml dichloromethane. The organic layer was washed with water, dried over MgSO₄ and the solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography, eluted with hexane/EtOAc (1:1 v/v) to give 8.43 g (91%) of pure product.

¹H-NMR (250 MHz, CDCl₃): δ = 2.24 (m, 2H, 2'-H), 3.32 (m, 2H, 5'-H), 3.72 (s, 6H, OCH₃), 4.01 (m, 1H, 3'-H), 4.47 (m, 1H, 4'-H), 6.24 (t, *J*₁=6.7Hz, 1H, 1'-H), 6.77 (d, *J* = 8.9 Hz, DMTr aromatic protons, 4H), 7.08 to 7.42 (m, 9H, DMTr aromatic protons), 8.07 (s, 1H, 6-H), 8.77 (s, N3-H, 1H).

¹³C-NMR (63 MHz, CDCl₃): δ = 40.3, 55.3(2C), 63.51, 68.94, 70.3, 85.05, 86.92, 87.5, 113.21 (4C) 125.9, 127.56 (2C), 127.68 (2C), 128.9 (4C), 132.35, 135.38, 144.19, 144.71, 150.1, 159.2 (2C), 160.52.

FD-MS: m/z calculated for C₃₀H₂₉IN₂O₇ = 656.46 g/mol; found: 656.2.

3.1.2. 5-[Dodec-1-ynyl] -5'-O-dimethoxytrityl-2'-deoxyuridine (**2**)

5'-O-(4,4'-Dimethoxytrityl)-5-iodo-2'-deoxyuridine (**1**) (8 g; 12.19 mmol) was dissolved in degassed anhydrous DMF (100 ml) and argon was bubbled through this solution for 10 min. Pd(Ph₃P)₄ (1.41 g; 1.22 mmol, 10 mol%) was added and argon was bubbled through the solution for another 5 min. Amberlite-IRA67 (20 g) was introduced, followed by addition of 1-dodecyne (6.1 g; 36.56 mmol) and copper(I) iodide (0.46 g; 2.44 mmol, 20 mol%). The mixture was stirred for 18 hrs at room temperature in the absence of light. The solid was filtered and washed with MeOH/CH₂Cl₂ 1:1 v/v (10 ml). The solvent was evaporated and the residue was purified by silica column chromatography, eluted with hexane/EtOAc (1:1 v/v) to give 5.96 g (70%) of pure product.

¹H-NMR (250 MHz, CD₂Cl₂): δ = 0.8 (t, *J* = 7.3 Hz, 3H, 1CH₃), 1.16 (s, 14H, 7CH₂), 1.5 (t, *J* = 6.6 Hz, 2H, 1CH₂), 1.99 and 2.20 (2 sets of multiplets, 2H, 2'H), 2.31 (m, 2H, 1CH₂), 3.25 (m, 2H, 1CH₂, 5'H), 3.48 (m, 2H, 3'-H and 3'-OH), 3.70 (s, 6H, 2OCH₃), 4.43 (m, 1H, 4'-H), 6.17 (dd, *J*₁ = 7.6 Hz, *J*₂ = 5.5 Hz, 1H, 1'-H), 6.74 (d, 4H, *J* = 8.8 Hz, DMTr aromatic protons), 7.25 (m, 9H, DMTr aromatic protons), 7.83 (s, 1H, H-6), 8.56 (s, N3-H, 1H).

¹³C-NMR (63 MHz, CDCl₃): δ = 14.3, 19.8, 23.1, 28.93, 29.73, 32.23, 41.70, 55.6, 63.99, 71.14, 72.69, 86.03, 87.00, 87.31, 87.9, 93.4, 94.60, 95.55, 101.1, 113.64, 116.8, 126.4, 127.25, 128.29, 128.40, 130.29, 135.92, 136.07, 145.26, 151.08, 153.08, 156.00, 159.45, 160.35.

FD-MS: m/z calculated for C₄₂H₅₀N₂O₇ = 694.86 g/mol; found: 694.4.

3.1.3. 5-(3'-Dodecyne)-5'-O-dimethoxytrityl-2'-deoxyuridine-3'[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] (**3**)

5-[Dodec-1-ynyl] -5'-O-dimethoxytrityl-2'-deoxyuridine (**2**) (5.64 g, 8.12 mmol) was dissolved in dry THF and reacted with *N*-diisopropyl-2-cyanoethyl-chlorophosphoramidite (2.31 g, 9.74 mmol) in the presence of diisopropylethylamine at room temperature. The reaction mixture was stirred for 3 hrs under argon atmosphere. The mixture was poured into saturated Na₂CO₃ solution and washed with water (3×) and brine (3×). The organic layers were combined and dried over MgSO₄. After evaporation of the solvent the product was dried under high vacuum to give **3** in quantitative yield. The product was used immediately for the solid phase DNA synthesis.

³¹P-NMR (100 MHz, THF-*d*₈): 149.65.

3.2. DNA Synthesis

Compound **3** (7 g) was dissolved in CH₃CN (52 ml) to give a concentration of 0.15 M. This solution of **3** was connected to DNA synthesizer prior to starting the DNA synthesis. The **UU11** synthesis was performed on a 50 μmol scale with an overall product yield of 16%, as calculated from the AIEX chromatogram.

P11 and **P22** DNA diblock copolymers with poly(propylene oxide) monobutyl ether (PPO, MW6800) were prepared according to a previous report.²

MALDI-TOF MS of **UU11**: 3,629 m/z ; calculated: 3,629 (figure S1)

3.4. Characterization of UU11 and P11/P22

3.4.1. MALDI-TOF mass spectroscopy

Matrix and sample preparation: 3-hydroxypicolinic acid (20 mg), picolinic acid (2 mg) and ammonium citrate (3 mg) were dissolved in 30% acetonitrile in water (0.5 ml). The mixing ratio was sample (20 μM) to matrix = 1 : 2 (v/v).

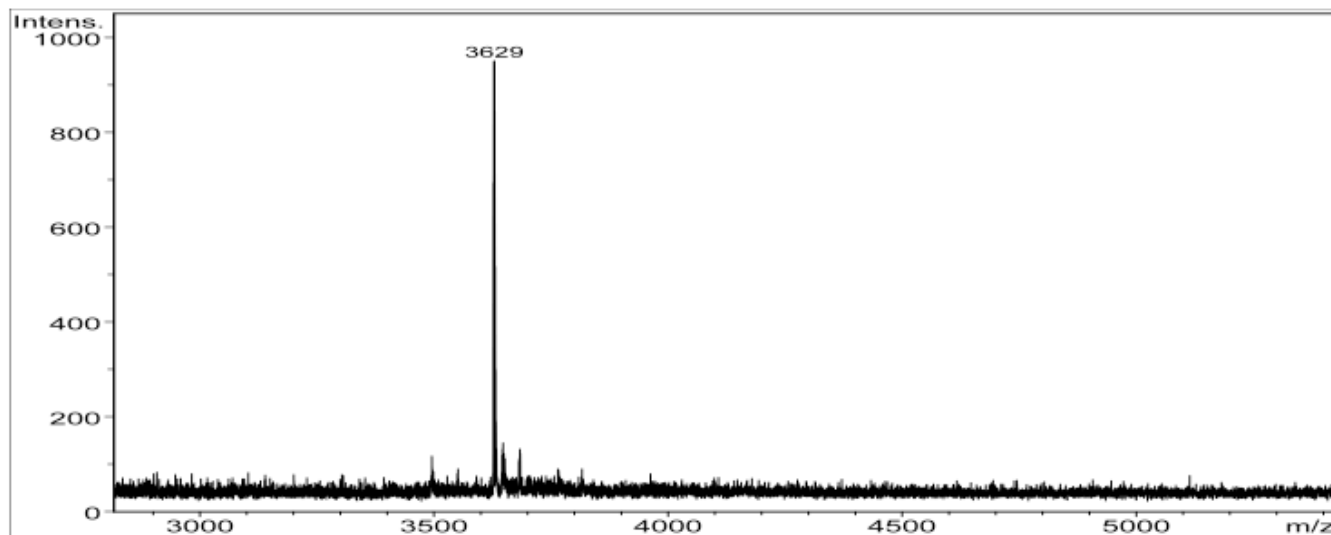
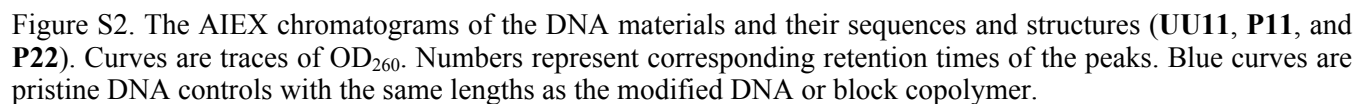


Figure S1. MALDI-TOF spectrum of **UU11**.

Analytical AIEX chromatography was performed using a HiTrap Q HP 1 ml column (GE Healthcare) and linear gradient elution with two elution buffers (buffer A: 25 mM Tris, buffer B: 25 mM Tris + 1.0 M NaCl).



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4. Characterization of DNA Micelles

4.1. DLS

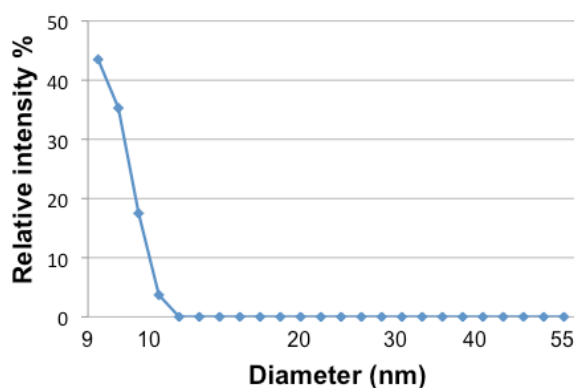


Figure S4. DLS size distribution of **UU11** (2 mg/ml in milli-Q water) analyzed by number.

4.2. AFM

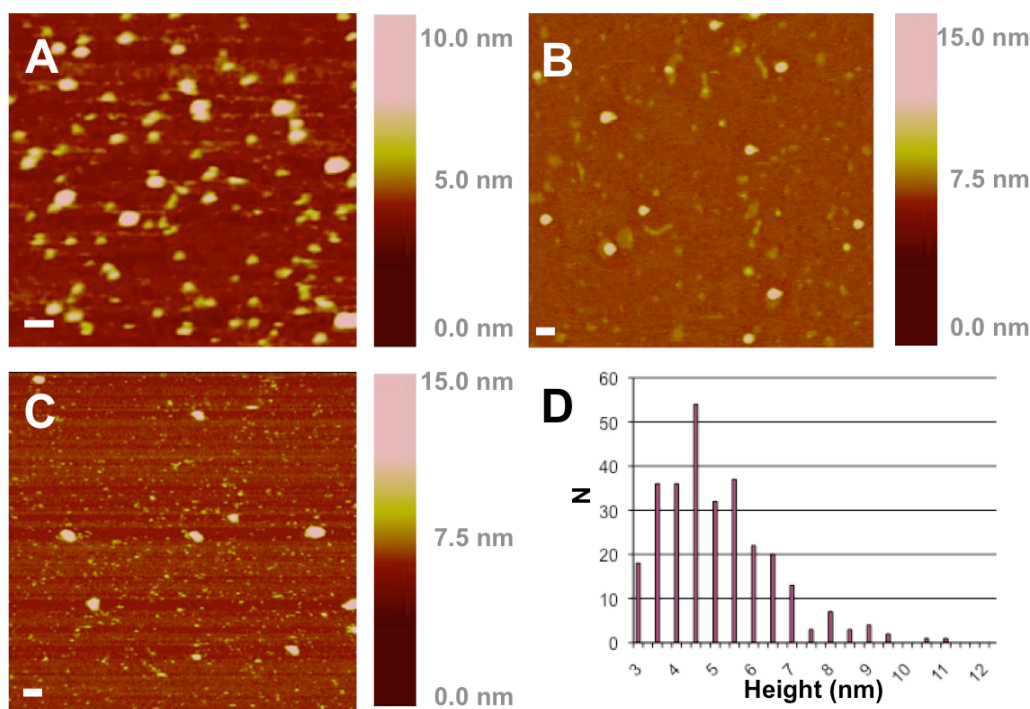


Figure S5. AFM images of the DNA micelles. (A) **UU11** in fluid cell. (B) **UU11** in air. (C) **P11** in fluid cell. (D) Size histogram of **P11**. The scale bars are 50 nm.

4.3. Calculation of the aggregation number (Z) of the UU11 micelle.

Calculation of Z using molar extinction coefficient (ϵ) of two dyes labeled DNA and CP: The molarity ratio of **UU11** : CP was calculated using the Beer-Lambert law, $A = \epsilon cl$. From the absorbance of ROX on complementary DNA (4.30 mAU, 50% hybridized) and Dylight647 on CP (85.5 mAU, 17% labeled), see elution bands at 1.28 ml in Figure S6A, a ratio of 1 : 4.82 was found. In a capsid of T = 2 geometry, which consists of 120 CPs, this corresponds to 25 single-stranded **UU11** molecules in the capsid-cavity. The micelle in the cavity

thus has an aggregation number of $Z = 24.9 \pm 2.8$. The weight ratio of nucleotides, including **UU11** and cDNA, in the cavity relative to VC was calculated to be 6%.

Geometrical estimation of Z: For the purpose of estimating the aggregation number of the **UU11** micelles, the single-stranded DNA strands of the corona were each assumed to be confined to spheres with a diameter of 2.7 ± 0.1 nm.^{3,4} For a micelle diameter of 10 nm as observed by DLS, this yields an approximate aggregation number of $Z = 23 \pm 2$, which is in good agreement with value calculated from measured molar extinction coefficients.

5. Preparation of Loaded Micelles

DiI or pyrene was dissolved in acetone. A drop of dye solution (400 $\mu\text{g/ml}$) was added to a tube and subsequently evaporated. A **UU11** micellar solution was added to the tube and the mixture was agitated overnight at room temperature.

50 Mol percent of ROX-ODN was hybridized with **UU11** in MgAc_2 (10 mM) buffer at pH 7.5.

6. Preparation of VCs

General procedure of VC formation: A DNA amphiphile and CP were mixed in a 1 : 2.3 molar (Table S1) in pH 7.5 capsid buffer and the mixture was allowed to form capsids for 1 h at 4 °C.

Table S1. Mixed volume and concentration of VCs

Amphiphile used for VC formation (molar ratio of the amphiphile : CP)	DNA amphiphile	Coat protein
Pyrene/ UU11 (1 : 2.3)	19.6 μL (208.58 μM)	100 μL (94.2 μM)
DiI/ UU11 (1 : 3.6)	8.1 μL (400 μM)	100 μL (148 μM)
ROX-ODN/ UU11 (1 : 2.3)	19.6 μL (208.58 μM)	90 μL (83.1 μM)

7. Size Exclusion Chromatography of Crude VC Mixtures

Size exclusion chromatography was performed on a FPLC system equipped with a Superose 6 column using the pH 7.5 capsid buffer. Multiple wavelength detection was applied during the elution. Fractions a and b of Figure S6B were measured by TEM, the images are shown in Figure 1.

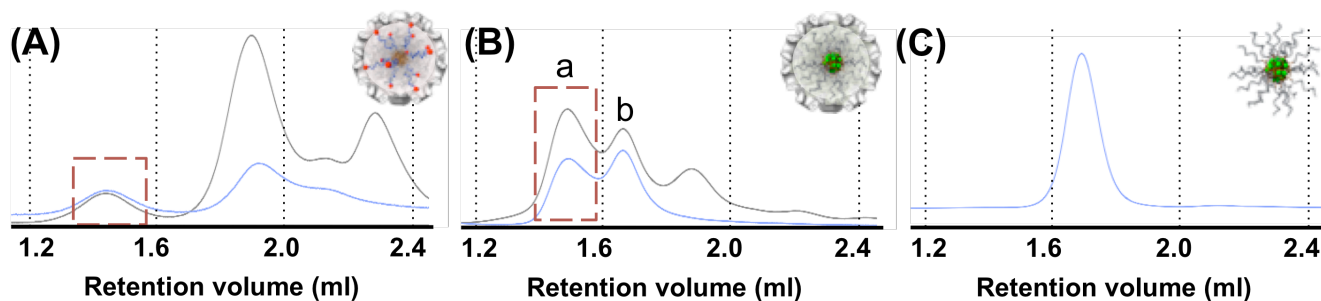


Figure S6. FPLC graphs of crude VC mixtures using the depicted loading strategies. (A) ROX-DNA hybridized **UU11**-VC. (B) DiI loaded **UU11**-VC. (C) DiI loaded **UU11** only. Ordinate is the normalized absorbance. Highlighted sections are fractions that were collected for further measurement. Black curves represent the protein absorbance at 280 nm, and the blue curves are the absorbance corresponding dye species (549 and 575 nm, respectively) loaded in the DNA micelle. The integrated area of Figure S6B shows that around 56% of the micelles are encapsulated into the VCs.

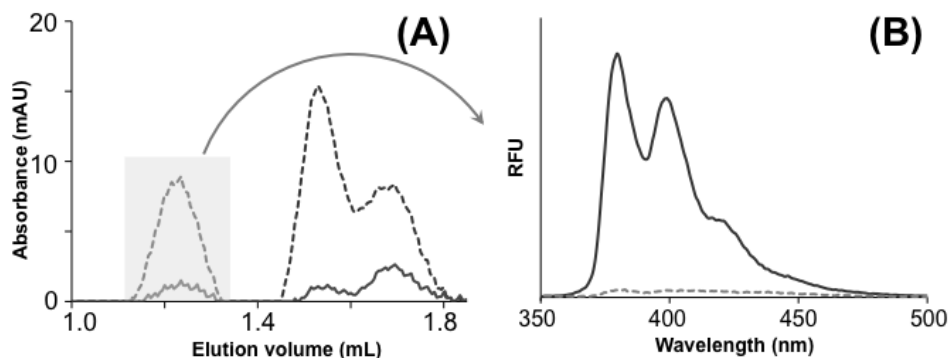


Figure S7. FPLC elugram and fluorescence spectra of pyrene-loaded **UU11-VC**. (A) Absorbance of protein (280 nm, dashed) and pyrene (342 nm, solid). (B) Fluorescence spectra ($\lambda_{\text{ex}} = 342$ nm) of the highlighted fraction (solid) and pristine CP (dashed).

8. Silver Protein Staining of in the Gel of **UU11-VC**

FPLC fractions of DiI loaded **UU11-VP** and non-encapsulated **UU11** were analyzed on an SDS-Page gel, and detected by silver staining. This showed the presence of capsid protein in the DiI loaded **UU11-VP** fraction at 1.4 mL (lane 3). Capsid protein dimers are known to elute at 1.8 mL (lane 5). They are subject to heavy degradation, while the wild-type capsid protein (wt CP) stored at pH 5.0 and the DiI loaded **UU11-VP** only show light degradation. Wt CP is known to degrade fairly quickly at pH 7.5, but not at pH 5.0. This is probably due to the fact that at pH 5.0 the *N*-terminus, which is prone to degradation, points inwards into the capsid and is thus more or less protected from the environment. Since the capsid proteins form around the micelle, the same mechanism probably protects the capsid proteins of the DiI loaded **UU11-VP** from degradation. Like the DiI loaded **UU11-VP**, the dimers that did not form around the micelle were left at room temperature for at least an hour, which speeds up the degradation process. The capsid proteins in the first lane were also kept at pH 7.5 for several hours, but they were stored in the refrigerator.

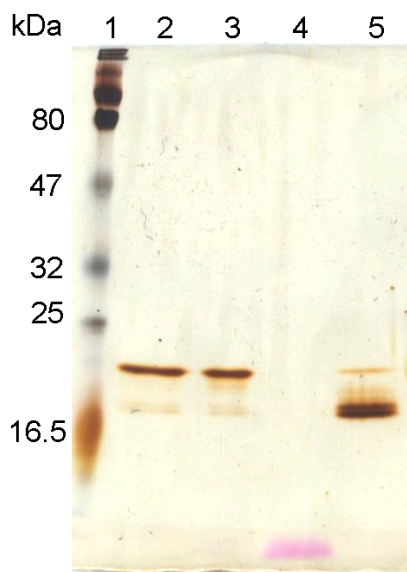


Figure S8. Silver protein stained gel of the **UU11** fractions from the SEC.

Table S2. Samples in the lanes of the gel (Figure S8)

Lane	Sample
1	Marker
2	Wt CP (wild type capsid protein)
3	UU11 , SEC fraction at 1.4 mL
4	UU11 , SEC fraction 1.6 mL
5	Wt CP: UU11 , FPLC fraction 1.8 mL

9. Additional Micrographs and Statistical Analysis of VCs

9.1. TEM results

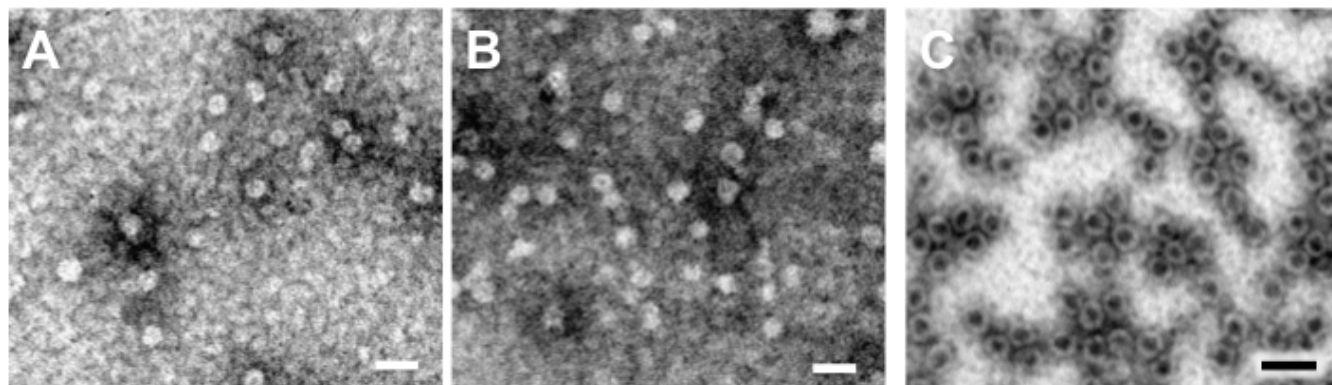


Figure S9. TEM images of **P11-VC** (A), **P22-VC** (B), and wild type empty capsid assembled at pH 5.0 (C). The scale bars are 40 nm.

9.2. Statistical analysis of the particle sizes observed by TEM

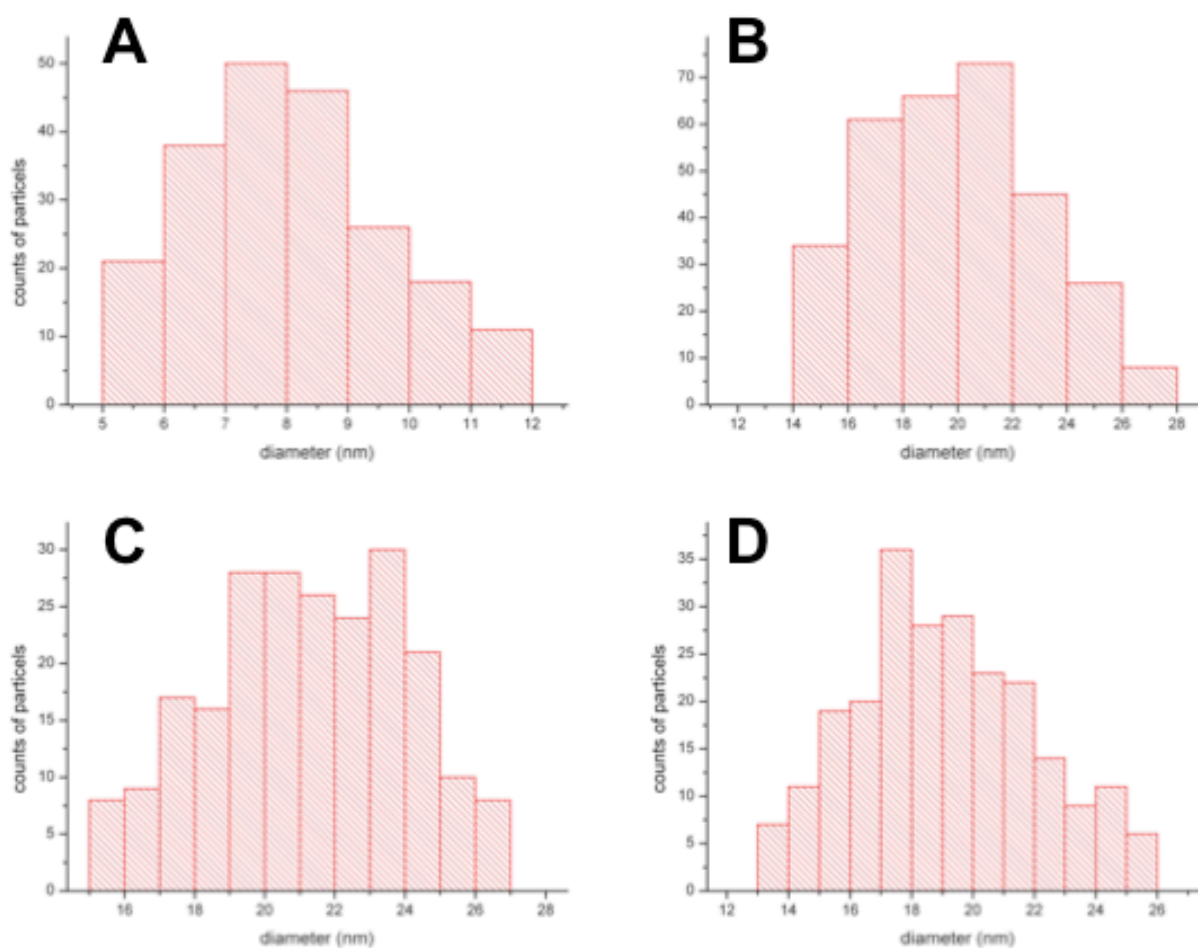


Figure S10. Size distribution histograms of **UU11** micelles (A, 8.1 ± 1.6 nm), **UU11** VC's (B, 19.9 ± 3.1 nm), **P11** VC's (C, 21.2 ± 2.8 nm), and **P22** VC's (D, 19.2 ± 3.5 nm) as determined from the TEM images.

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